Molecular Biology Reports (2023) 50:1281–1291
https://doi.org/10.1007/s11033-022-08084-8

ORIGINAL ARTICLE

Evaluation of osteogenic induction potency of miR-27a-3p in adipose tissue-derived human mesenchymal stem cells (AD-hMSCs)

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Received: 1 September 2022 / Accepted: 1 November 2022 / Published online: 30 November 2022

Abstract

Background Bone tissue as a dynamic tissue is able to repair its minor injuries, however, sometimes the repair cannot be completed by itself due to the size of lesion. In such cases, the best treatment could be bone tissue engineering. The use of stem cells in skeletal disorders to repair bone defects has created bright prospects. On the other hand, changes in the expression level of microRNAs (miRs) can lead to the commitment of mesenchymal stem cells (MSCs) to cell lineage. Many studies reported that post-transcriptional regulations by miRNAs are involved in all stages of osteoblast differentiation.

Method After the preparing adipose tissue-derived mesenchymal stem cells, the target cells from the third passage were cultured in two groups, transfected MSCs with miR-27a-3p (DM.C + P) and control group. In different times, 7 and 14 days after culture, differentiation of these cells into osteoblast were measured using various techniques including the *ALP* test and calcium content test, Alizarin Red staining, Immunocytochemistry technique (ICC). Also, the relative expression of bone differentiation marker genes including Osteonectin (*ON*), Osteocalcin (*OC*), RUNX Family Transcription Factor 2 (*RUNX2*), Collagen type I alpha 1 (*COL1*) was investigated by real-time RT PCR.

Results In comparison with control groups, overexpression of miR-27a-3p in transfected cells resulted in a significant increase in the expression of bone markers genes (*ON*, *OC*, *RUNX2*, *COL1*), alkaline phosphatase (*ALP*) activity, and calcium content ($p < 0.05$). In addition, the results obtained from ICC technique showed that osteocalcin protein is expressed at the surface of bone cells. Furthermore, the expression of *APC*, as a target of miR-27a-3p, decreased in transfected cells.

Conclusion Our data suggest that miR-27a-3p may positively regulates adipose tissue-derived mesenchymal stem cell differentiation into bone by targeting *APC* and activating the Wnt/b-catenin pathway.

Keywords miR-27a · Osteogenic differentiation · *APC*

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Abbreviations

WNT	Wnt/β-catenin pathway
TGF-β	TGF-beta signaling
BMP	Bone morphogenetic proteins
FGF	Fibroblast growth factors
OC	Osteocalcin
ON	Osteonectin
COL1	Collagen type I alpha 1
RUNX2	RUNX Family Transcription Factor 2
MiRs	MicroRNAs
MSC	Mesenchymal Stem Cells
ADMSCs	Adipose-derived mesenchymal stem cells
APC	Adenomatous polyposis coli
hBMSCs	Human bone marrow stem cells
DMEM	Dulbecco's Modified Eagle Medium
ICC	Immunocytochemistry
ALP	Alkaline phosphatase

Introduction

Bone as an essential tissue in the human body is responsible for protecting other organs, producing blood cells, and regulating hormones [1]. Every year, millions of people around the world suffer from bone fractures and injuries [2]. Bone tissue can repair its minor injuries, due to its biological stimuli for bone formation, including different cell types, different signaling pathways and various transcription factors [3].

There are several treatments available for bone injuries [4]. Although bone grafting is one of the most common surgical methods for orthopedic bone strengthening and reconstruction [5], it has many limitations, therefore new stem cell-based therapies are being developed. The use of stem cells in skeletal disorders to repair bone defects has created bright prospects [6].

Mesenchymal stem cells (MSCs) are a group of pluripotent stem cells [7]. These cells were first identified by Friedenstein in 1968 as fibroblast-like adhesion cells [8] and have the ability to differentiate into the lineage of cartilage, bone, fat, myogenic, and hematopoietic supportive cells [9, 10]. MSCs can be isolated from various biological sources such as adipose tissue [11], dental tissues [12], [13], placenta and fetal membrane [14], limb bud [15], and potentially many other tissues. Because of their potential capabilities to differentiate into osteoblasts [16], [17], they are on the top of the list of advanced treatment of many diseases [18] such as diseases related to bone metabolism [19].

On the other hand, MicroRNAs (miRs) have been reported in various studies as post-transcriptional regulators of gene expression, which play an essential role in regulating stem cell differentiation [20, 21]. MiRNAs are a small group of non-coding, single-stranded RNAs with 18 to 25 nucleotides long [22]. In humans, miRNAs regulate more than 30% of protein-coding genes. Transfer of miRNA to the target tissue can induce specific settings that inhibits or promotes cell growth and proliferation, associated with determining cell fate [23]. The use of MicroRNAs has led to many advances in bone repair techniques based on MSCs [24].

Owing to the importance of miRNAs in osteogenesis, where there are many involved miRs, amongst them, miR-27a-3p was selected. Because overexpression of miR-27a-3p in patients with osteosarcoma [25] and gastric cancer is associated with poor prognosis, it is considered as an oncogene [26]. Some articles have also suggested that miR-27a-3p plays an important role in the development of the tumor [27–29]. Furthermore, research has shown that the overexpression of miR-27a-3p increases the proliferation and invasion of colon cancer cells and then can be effective in the treatment of bone joints [30]. To achieve the role of miR-27a-3p during osteoblast differentiation, we used bioinformatics analysis. The *APC* gene was identified as one of the downstream targets of miR-27a-3p and as an inhibitor of the Wnt/b-catenin signaling pathway. The present study was performed to evaluate the osteogenic induction potency of miR-27a-3p in adipose-derived human mesenchymal stem cells (AD-hMSCs). The result identified miR-27a-3p acts as a positive regulator of bone differentiation by suppressing *APC* and activating the Wnt/b-catenin signaling pathway in AD-MSCs.

Materials and methods

Culture of AD-hMSCs and their transfection

MSCs have been shown to differentiate into bone cells within an osteogenic differentiation medium during a 21-day process [31]. Therefore, in this study, two groups (MSCs with miR-27a-3p (DM.C + P) and a control group (DM.C)) were examined to compare the process of differentiation in the presence and absence of microRNA. AD-MSCs, as described previously, were isolated from adipose tissue (Imam Hospital, Tehran, Iran) [32]. Some cells were frozen and stored in a nitrogen tank for future studies. Fresh cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) low glucose medium with 10% fetal bovine serum (FBS) and 1% Pen/strep (complete medium) and incubated at 37 °C and 5% CO2. The fourth passage of cells was planted in 48 well plates (12×10^3 cells per well). When 80% of the surface of a culture vessel was covered with cells, miR-27a-3p was transfected into AD-MScs. After 12 h, the complete medium was replaced with an osteogenic differentiation medium. Seven days later, the transfected cells were harvested to evaluate the expression of the miR-27a-3p gene (Sequences of primers are listed in Table 1).

Table 1 miRNA RT Stem-loops and Primers. (Snord was used as a reference for miRNA)

hmiR-27a-RT	5′-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACGCGGAA-3′
hmiR-27a F	5′-CCGTTCACAGTGGCTAAG-3′
Snord-47 RT	5′-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTC-3′
Snord-47 F	5′-ATCACTGTAAAACCGTTCCA-3′
Universal Rev	5′-GAGCAGGGTCCGAGGT-3′

RNA extraction and quantitation

For the extraction of RNA, the Pars Tous extraction kit (Pars Tous, Mashhad, Iran) was used, and according to the manufacturer's instructions, total RNA was extracted. RNA samples were stored at − 70 °C. For cDNA synthesis from RNA, Pars Tous cDNA synthesis kit (Pars Tous, Mashhad, Iran) was used, and qPCR was performed by SYBR Green method in the final volume of 12 µl. The list of primer sequences used in Real-time PCR is given in Table 1. The expression of bone markers gene, including *OC*, *ON*, *RUNX2*, *COL1*, and *APC* as the target gene of miR-27a-3p, was assessed in comparison with the B-actin reference gene (Table 2).

Alizarin red staining

At the end of the 21-days period of bone differentiation, Alizarin red staining was used to evaluate the presence of calcium deposits in the differentiation group compared to the control group. For this purpose, the cells were washed three times with Phosphate-Buffered Saline (PBS) then fixed with 4% formaldehyde for 15 min. After re-washing, they were stained with Alizarin red staining for 13 min. The presence of calcium deposits was investigated using an inverted microscope.

Alamar blue assay

-AD-MSC cells were planted in four different groups, 1. Transfected cells group with miR-27a-3P and commercial osteogenic differentiation medium (DM.C + P), 2. Control group cultured in commercial osteogenic differentiation medium (DM.C), 3. Control group cultured in manufactured osteogenic differentiation medium in vitro (DM.M) and 4. Control group cultured in DMEM medium (DMEM). Each group in triplicates were sets in 96-Well Plate. The cells of the group (DM.C + P) were transfected. After 3-time lapse (24 h, 3 and 5 days), the cell viability of all groups was measured with resazurin assay. At first 200 µl of complete medium and 50 µl of resazurin was added to each well, after incubating for 3 h in darkness, Optical Density (OD) was read in the Epoch Plate Reader (BioTek, United States) at 570/600 nm.

Table 2 Primer sequences used in this study

Target gene	Primer sequence
B-Actin	F: 5′-TCATGAAGTGTGACGTGGACATC-3′ R:5'-CAGGAGGAGCAATGATCTTGATCT-3′
RUNX2	F: 5′-GCC TTC AAG GTG GTA GCC C-3′ R: 5'-CGT TAC CCG CCA TGA CAG TA-3′
OC	F: 5′-GCA AAG GTG CAG CCT TTG TG-3′ R: 5′-GGC TCC CAG CCA TTG ATA CAG-3′
ON	F: 5′-AGG TAT CTG TGG GAG CTAATC-3′ R: 5′-ATT GCT GCA CAC CTT CTC-3′
COL1	F: 5′-TGG AGC AAG AGG CGA GAG-3′ R: 5′-CAC CAG CAT CAC CCT TAG C-3′
APC	F:5′- ACGAGCACAGCGAAGAATAGC-3′ R: 5′-TGTAGTTGAACCCTGACCATTACC-3′

Biochemical tests

ALP activity

After two time periods (7, 14 days) Alkaline Phosphatase (*ALP*) activity was measured for transfected cells and control groups. According to the instructions of the Pars Azmoun alkaline phosphatase quantitative detection kit (Pars Azmoun, Tehran, Iran), cell lysis was done with RIPA solution for 10 min and then centrifuged for 15 min (15000 rpm, 4 °C). R1 and R2 reagents were added to 20 µl of sample, and Optical Density was read at 405 nm in time intervals of 0,1,2,3 min.

Calcium content assays

Calcium content was measured for transfected cells and control groups after two time periods (7, 14 days) according to the kit instructions (Pars Azmoun, Tehran, Iran). The steps are summarized as follows: Lysis of cells with 0.6 N HCL, incubation for 5 min, adding R1 reagent to 10 µl of sample, incubation and finally reading the Optical Density at 650 nm by spectrophotometer.

Immunocytochemistry (ICC) technique

In accordance with the kit instructions, 14 days after cell transfection, cell fixation was performed with 4% paraformaldehyde for 10 min, then primary antibody fluorescein isothiocyanate (FITC) was added and incubated in the darkness for 1 h. Cells were washed three times with phosphate buffer Saline-Tween 20 (PBST) to remove unbound antibodies. Then, 4, 6 diamidino 2 phenylenole (DAPI) drops, were added to mark the nucleus of the cells (1: 1000). The result was observed under an inverted microscope.

Bioinformatic prediction and *in-silico* study

Identification of miRNA target genes was an essential task. In this study, prediction sites such as Tarbase (Tarbase v8), miRDB, and mirPath were used to select the appropriate target gene for miR-27a-3P. The *APC* gene then was nominated as the possible target of miR-27a-3p through the Wnt signaling pathway.

Statistical analysis

Tests were done for each sample at least in three replications. The analysis of data was done using one-way ANOVA test by Graphpad Prism 9 Software (Graphpad Software, Inc, La JOLLA, California). P values ≤ 0.05 were considered statistically significant. The $\Delta\Delta CT$ method was used to measure the relative expression of qPCR data. Data are shown as mean ± SD.

Results

AD-MSC differentiation potential

About ten days after the onset of differentiation, changes in the shape of the cells and differentiation into the bone were observed. Cells were cultured in bone differentiation medium for 21 days. The specialization of cells and the formation of mineral matrix around them was shown by Alizarin Red staining in the form of red masses. As mentioned, adipose-derived stem cells have the ability to differentiate into bone and adipose tissue. To test this feature, we also did Oil Red Staining to evaluate the differentiation of cells into adipocytes. For this purpose, after a 21-day period, cultured cells in adipose differentiation medium were stained with Oil red. Because the fat droplets were visible in the cells seven days after differentiation, the fat droplets turned red in this staining.

The results highlighted that mesenchymal cells isolated from human adipose tissue in vitro could be differentiated into at least two cell lines. The results of the alizarin red and Oil Red staining are depicted in (Fig. 1A, B).

Confirmation of AD-MSCs transfection

After extracting the plasmid containing miR-27a-3P from the bacteria, they were transfected into target cells. To check the presence of the plasmid in the transfected cells, at the first stage, 48 h after transfection, the cells were examined under fluorescence microscopy. In the second stage, the relative expression of miR-27a-3p was measured using a stem-loop real-time PCR and then was compared in the transfected and control groups (DM.C) [33].

Due to the presence of the GFP gene in the pCDH plasmid, observation of green light in about 80% of the cells indicated that most cells were able to receive miR-27a-3p (Fig. 1C, D, E). Also, the expression of miR-27a-3P in the transfection group showed a significant increase compared with the control group.

Evaluation of cellular survival in different groups

The percentage of cell survival was studied in 4 different groups and three periods (1,3 and 5 days). As shown in (Fig. 2A) there was no significant difference between the 4 groups in days 1 and 3. Although there was a significant difference between the control group (DM.C) and transfection group (DM.C + P) in day 5, according to the results, it can be postulated that the expression of the miR-27a-3p gene in the transfected cells was not toxic, and the cells continued to grow.

Biochemical tests

Calcium and alkaline phosphatase tests are biochemical tests performed in this study that examined two differentiation features of bone cells.

Calcium content assays

As shown in (Fig. 2B) evaluation and comparison of calcium data in differentiation groups (DM.C, DM.C + P) on different days (7 and 14 days) demonstrated that the increasing trend of calcium levels along with the progression of bone differentiation, showed a significant increase. The Calcium content in the transfection Group (DM.C + P) was substantial.

ALP assays

The activity of *ALP* enzyme is one of the indicators of bone differentiation. As shown in (Fig. 2C) alkaline phosphatase activity was significantly increased on day 14 in the transfection group (DM.C).

Investigating the expression of osteocalcin protein

In order to accurately evaluate the differentiation of AD-MSCs cells into osteoblasts, osteocalcin protein expression was examined by ICC technique. The results represented that osteocalcin protein was expressed on the surface of bone cells (Fig. 3B, C).

RT-qPCR analyses

Changes in the expression level of bone marker genes (*OC*, *ON*, *COL1*, *RUNX2*) were evaluated using real-time RT PCR between two groups (DM.C, DM.C + P) on days 7 and 14. The results indicated a significant increase in the expression of all gene markers in AD-MSCs transfected with miR-27a-3p. On day 7, the *OC* gene exhibited the

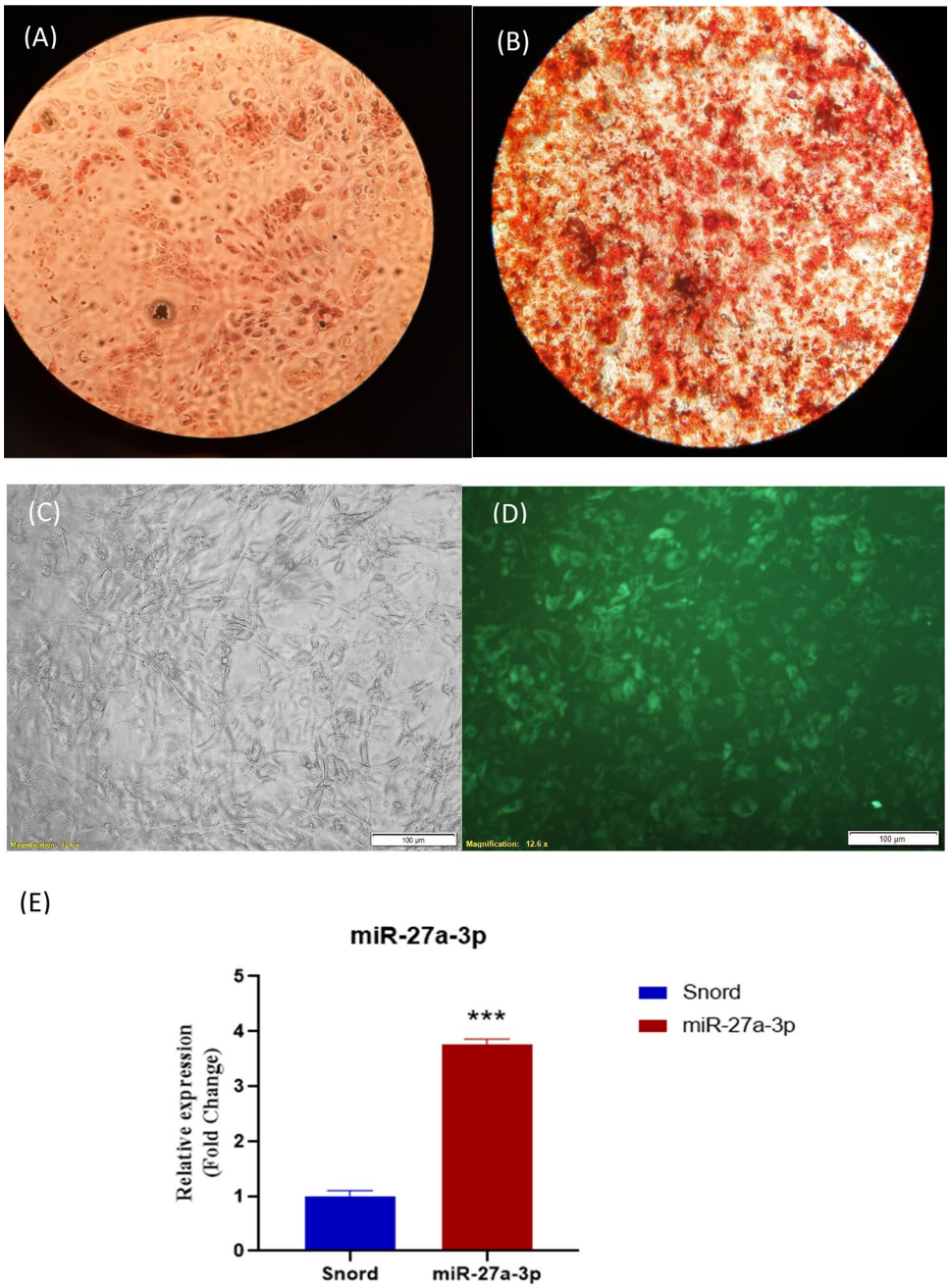

Fig. 1 Osteogenic differentiation AD MSCs under osteogenic differentiation medium after 21 days **A** with Oil Red Staining **B** Alizarin Red Staining. Transfection of ADMSC cells using vector LTR. **C** AD-MSCs by light inverted microscope. **D** miR-27-3p-transduced AD-MSCs by fluorescent microscope (magnification is 100×). **E** Relative expression of miR-27a-3p in the transfected group (DM.C + P) compared with the control group (DM.C). (***P-value < 0.000)

highest expression level compared to the other gene markers (Fig. 3A).

APC is the direct target of miR-27a-3p

Based on real-Time PCR results the expression of *APC* gene showed that this gene was reduced in transfected cells. The results of in silico studies also showed that *APC* is directly targeted by miR-27a-3p, which is consistent with the results presented in (Fig. 4).

Discussion

Mesenchymal stem cells are pluripotent cells that can differentiate into connective tissue cell types such as osteoblasts, chondrocytes, adipocytes, and myoblasts [34]. In this study, we assessed the osteogenic induction potency of miR-27a-3p in AD-hMSC through transfection by miR-27a-3p. The differentiation process of the transfected cells was then examined by various tests, including Alizarin red staining, *ALP* activity, and Calcium content assays for

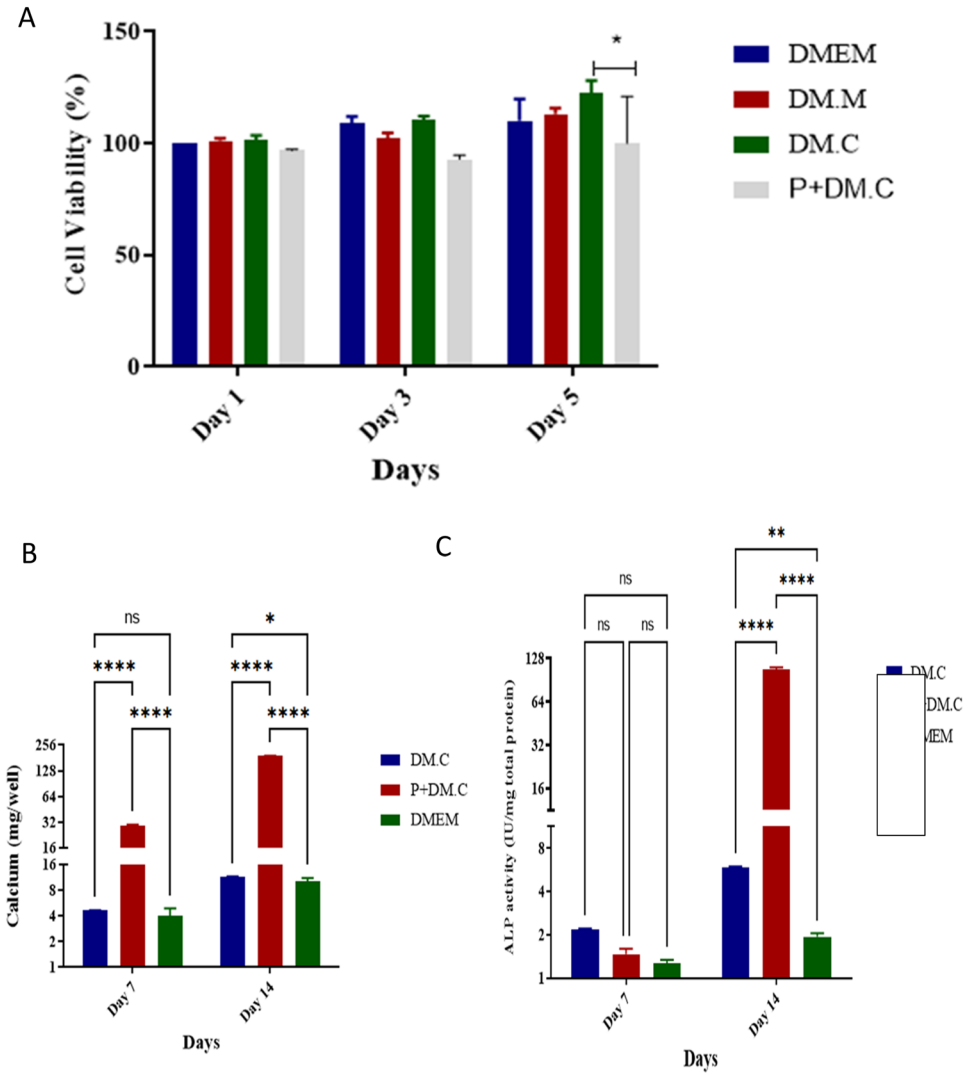

Fig. 2 **A** Viability of the cells isolated from adipose tissue (AD-MSCs) in four different groups by Alamar Blue Assay. The significant difference between the groups is indicated with an asterisk (*P < 0.05). **B** calcium content (*p-value < 0.05, ****p-value < 0.0001). **C** Alkaline phosphatase activity (.**p-value > 0.01, ****p-value < 0.0001)

14 days. To find the effect of miR-27a-3p on bone differentiation of transfected cells, we also investigated the bone marker genes expression including *OC*, *ON*, *RUNX2*, and *COL1*. Additionally, Immunocytochemistry was used to verify the results.

MSCs differentiation into other cell lines is influenced by various extracellular factors such as signaling pathways, miRNAs as well as some transcription factors and growth factors [35]. Different signaling pathways play a vital role in MSCs differentiation and Wnt/b-catenin pathway is one of the most important signaling pathways, which is crucial for the differentiation of human mesenchymal stem cells into osteoblasts [36, 37]. This pathway also plays a central role in the early stages of osteogenesis, while it is down-regulated in differentiated cells [38].

In early stages of osteogenesis, activation of the Wnt signaling pathway leads to the accumulation of beta-catenin in cytoplasm (Fig. 5). After transferring to the nucleus, it will be bound to LEF/TCF transcription factors [39]. Its interaction with other transcriptional regulators like RUNX2 and OSX, which are essential transcriptional factors in osteogenesis [40], eventually leads to transcription of bone differentiation genes including type I collagen, osteopontin, bone sialoprotein, and osteocalcin [41]. The expression of certain transcription factors, cell surface markers, and extracellular matrix products are indicators of the transition from an MSC to a mature osteoblast [42]. Runx2 and Osterix, two essential transcription factors, are first induced to drive MSCs to osteogenesis [43]. These transcription factors first induce the release of alkaline phosphatase (*ALP*) and type 1 collagen (*COL1*), then osteonectin (*ON*) and osteocalcin (*OC*) in the late stages of maturation, causing osteoprogenitors to develop into adult osteoblasts [44]. Osteocalcin, one of the most abundant non-collagenous proteins, is mainly found in osteoblasts [45]. The majority of the *OC*, released by osteoblasts, is absorbed into the organic matrix, which eventually ossifies into bone [46]. *OC* is commonly used as a marker of bone

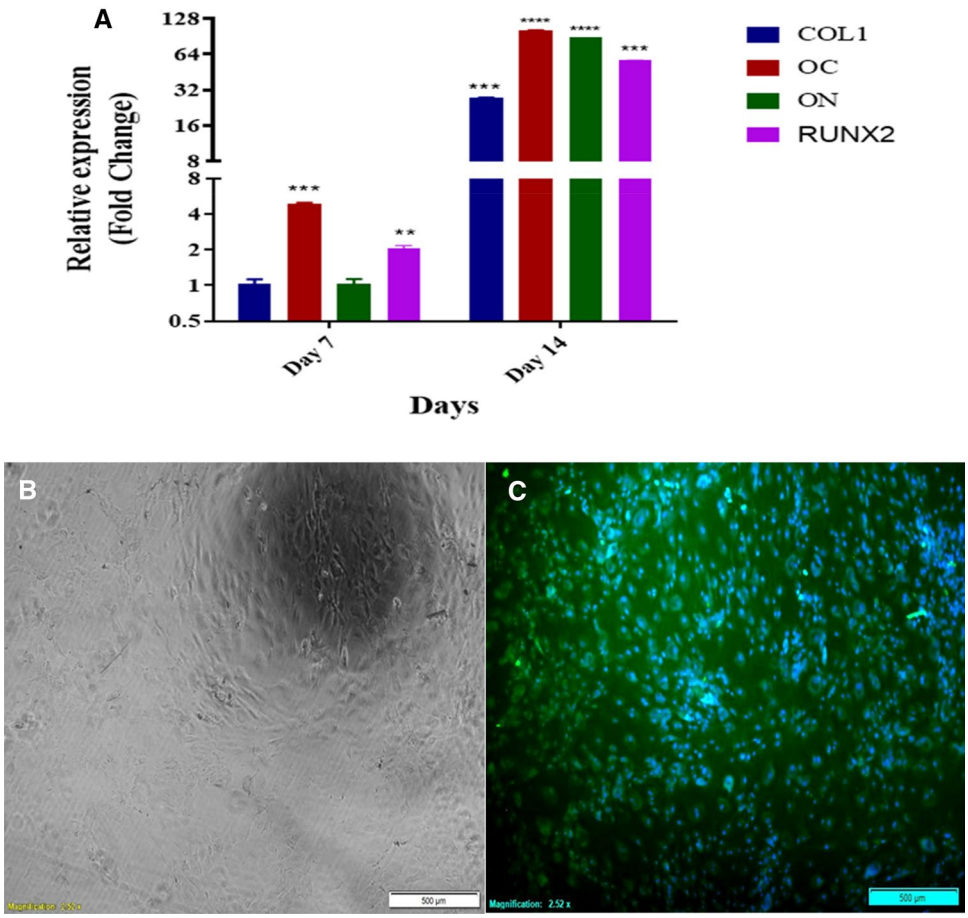

Fig. 3 Osteogenic related genes and protein expression**. A** Relative expression of bone markers gene including *OC*, *ON*, *COL1*, *RUNX2* in the transfected group (DM.C + P) compared with the control group (DM.C). (**P < 0.01, ***P < 0.001, ****P < 0.0001). DAPI was used to label the cell nuclei. **B** AD-MSCs by light inverted microscope. **C** The protein expression level with immunocytochemical analysis

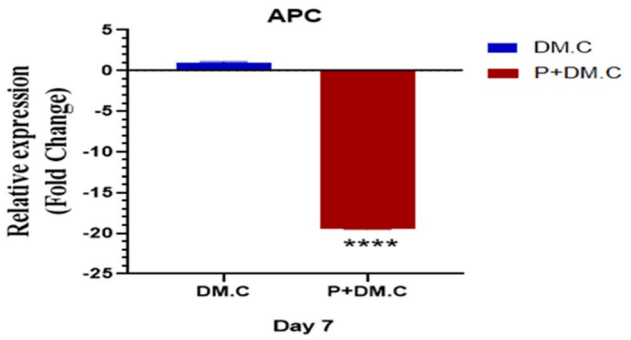

Fig. 4 Relative expression of *APC* gene in the transfected group (DM.C + P) compared with the control group (DM.C). (****P < 0.0001)

development, and measurements of direct bone growth correlate with the concentration of *OC* [47].

MicroRNAs are another essential factors in this process. There is ample evidence suggesting that post-transcriptional regulation of gene expression, mediated by microRNAs, plays an important role in controlling osteoblasts differentiation. MiR-29a [48], MiR-26a [49], MiR-217 [50], MiR-10b [51], MiR-181a [52] and MiR-322 [53] are the main players in this regard.

As a result, it is concluded that miR-27a-3p promotes osteogenic differentiation by mediating Wnt signaling pathway. To support this idea, we can point to the similar results in other studies. MiR-27a-3p plays a positive role in the osteoblastic differentiation of hFOB cells by targeting the *sFRP1*, an extracellular antagonist of the Wnt signaling pathway, [54]. Similarly, the positive role of miR-27a-3P in hFOB cells is exerted by downregulating the inhibitor of the Wnt pathway, *APC* [55].

On the other hand, the results of the study published by Hassan et al., indicated that miR-27a is a negative regulator in early stages of bone differentiation in MC3T3-E1 cells, which delays osteoblast differentiation through *SATB2* gene downregulation [56]. Also, despite the fact that miR-27a is essential for bone formation, it could reduce the osteogenesis in hBMSC cells by suppressing *Pex7*, *GCA*, and *APL* in early stages [57]. Recently, some results in several studies showed that despite the positive regulatory role of miR-27b in the overall differentiation process of induced pluripotent stem cells (hiPS) via the BMP signaling pathway, this microRNA exerts a negative role in early stages of differentiation [58]. Likewise, studies that investigated the role of miR-27a-3p in

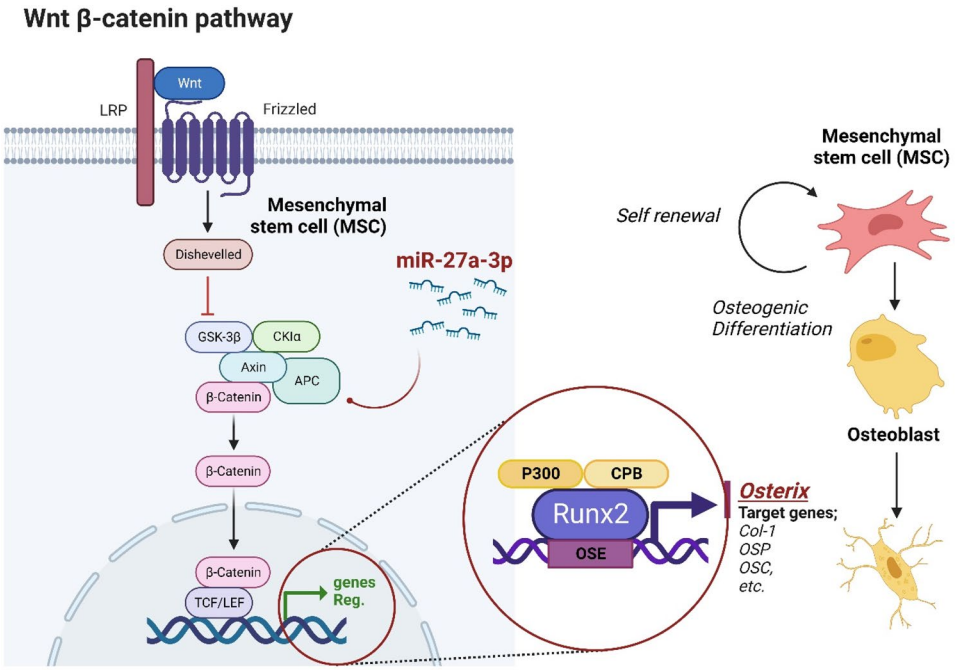

Fig. 5 miR-27a Significance in Wnt Signaling pathway**.** After the accumulation of b-catenin and transferring to the nucleus, transcription of the osteogenesis target genes is prompted by activated transcription factors such as RUNX2, OSX.

the miR-23a~27a~24–2 cluster have mentioned that over-expression of miR-27a is associated with decreasing osteogenesis [56, 59, 60]. There has to be a reason behind this contradiction. One possibility is that because miR-27a has been examined in the cluster form not by itself alone.

So overall we can conclude that over-expression of miR-27 in different cells can be followed by the effect of miR-27 on downstream genes in signaling pathways which occurs at intervals of 6 to 7 days and results in triggering signaling cascades. These data suggest that miR-27a is a negative regulator in the early stages of osteogenesis.

Adenomatous polyposis (*APC*) is an indirect inhibitor of osteoblast differentiation that acts by suppressing the Wnt/b-catenin pathway [61]. B-catenin, a crucial protein that activates Wnt signaling, accumulates due to the suppression of *APC* expression [62]]. Activation of b-catenin following *APC* knockdown induces transcription of Wnt target genes, including *COL1*, *OSP*, *OSC*, and others, leading to osteoblast formation [63]. Therefore, we also studied *APC* gene expression. The results of RT-qPCR pointed out that miR-27a-3p, by targeting the *APC* gene, may promote Wnt signaling pathway more powerfully, and launches the main genes for bone differentiation. Past evidence demonstrated that *APC* is targeted by miR-27a-3p in MC3T3-E1 cells and thus promotes bone differentiation through the Wnt signaling pathway [64].

The data in the present study suggested that post-transcriptional regulation, made by microRNA, plays an important role in osteoconductivity. By transferring miR-27a-3p to MSCs and comparing the results with the control group, we observed different outcomes in cell fate. Based on these findings, it can be concluded that changes in microRNA levels can alter the fate of the cell (here, changes in miR-27a-3p expression led to the differentiation of adipose tissue-derived mesenchymal stem cells into osteoblasts). The results may also support that microRNAs with a role in differentiation of mesenchymal stem cells into osteoblasts could pave the way for new therapeutic options in skeletal disorders such as osteoarthritis (OA). One of the main causes of chronic disability is osteoarthritis (OA) [65]. Rocha et al. reported that the diagnosis and prognosis of OA can be accurately predicted using biomarkers such as microRNAs [66]. They showed that various soluble biomarkers related to joint metabolism could be used as OA indicators [66]. The association of miRNAs in cancer progression as prognosis biomarkers is a focus of attention today [67]. For example, bladder cancer progression is influenced by the regulation of miRNA expression by lncRNAs and circRNAs [68]. Progression of bladder cancer is inhibited by onco suppressor miRNA overexpression [69]. In addition, miR-27a-3p has been documented to function as a tumor suppressor in human malignancies [71]. miRNAs have been discovered as a potential therapeutic target for cancer [72, 70].

Conclusion

Overall, this study identified that overexpression of miR-27a-3p can accelerate and facilitate osteogenesis in AD-MSCs with the effect on the cell signaling network. In other words, by targeting *APC* it can activate the Wnt/b-catenin signaling pathway. Understanding the mechanisms involved

in osteoblast differentiation, which are regulated by specific microRNAs, is essential for developing new therapies for bone disorders.

Funding The authors would like to appreciate Research Deputy of Mashhad University of Medical Sciences for supporting this study (IR.MUMS.REC.1400.329).

Declarations

Conflict of interest The authors declare that they have no competing interests.

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable.

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